Summer 2015

Structural Studies of Blood Coagulation Factor VIII in Protein Complexes

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Structural Studies of Blood Coagulation FVIII in Protein Complexes

By

Michelle E. Wuerth

Accepted in Partial Completion

Of the Requirements for the Degree

Master of Science

Kathleen L. Kitto, Dean of the Graduate School

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MASTER’S THESIS

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Michelle Wuerth

July 31, 2015
Structural Studies of Blood Coagulation Factor VIII in Protein Complexes

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Michelle E. Wuerth
July 2015
Abstract

A deficiency in blood coagulation factor VIII (fVIII) is responsible for the inherited bleeding disorder hemophilia A, which affects approximately 1 in 5000 males. The development of inhibitory antibodies is a significant issue faced by hemophilia A patients receiving therapeutic infusions of fVIII. The C-terminal C2 domain of fVIII has been shown to be highly immunogenic and the site of binding for numerous antibodies of both the classical and non-classical classifications. A detailed understanding of the structural components involved in C2-antibody binding interactions is vital for the development of improved therapeutics for hemophilia patients. Here we present the structure of the classical antibody 3E6 bound to human C2 at 2.6 Å resolution. Previous studies have suggested that pairs of classical and non-classical antibodies may exhibit binding cooperativity. Comparison of the C2:3E6 structure with a previously determined ternary structure of C2 bound simultaneously to both 3E6 and the non-classical antibody G99 reveals that cooperativity is not the result of dramatic conformational changes at the binding interface. Rather, changes in B-factor ratios between the C2 complexes and the isolated C2 domain suggest that dynamic changes upon 3E6 binding may lead to more favorable binding of a second antibody on the protein’s opposite face. Studies of the fVIII C2 domain were then extended to attempts to understand the structural basis for the interactions between fVIII and its circulatory partner von Willebrand
factor (vWF), which is crucial for maintaining fVIII levels in plasma. The inability for vWF to bind fVIII is the basis for another blood coagulation disorder, von Willebrand’s disease type 2N, which can lead to hemophilia-type levels of fVIII in the blood and subsequent bleeding episodes. Previous publications have suggested a role for C2 in the binding of vWF. Attempts were made to formulate protein complexes between two vWF domain mutants, TIL'E' and D'D3, and the human C2 domain, but were ultimately unsuccessful. This outcome corroborates recent publications suggesting that the fVIII C1 domain may play a more active role in binding vWF than the C2 domain. In order to further study this possibility, complexes were formed between recombinant B-domain deleted fVIII and TIL’E’ or D’D3 and X-ray crystal trials initiated. Hopefully, greater understanding of the structural interactions between fVIII and other proteins will lead to improved therapies for patients suffering from fVIII-related diseases.
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List of Abbreviations

Ab – antibody
AMP – ampicillin
AP – alkaline phosphatase
BLI – biolayer interferometry
BME - β-mercaptoethanol
BSA – bovine serum albumin
CAPS – 3-(cyclohexylamino)-1-propanesulfonic acid
CHES – N-cyclohexyl-2-aminoethanesulfonic acid
CDR – complementarity determining region
CTCK – c-terminal cysteine knot domain
CV – column volumes
DsbC – disulfide bond isomerase
EM – electron microscopy
F_{AB} - antigen binding fragment (of an antibody)
F_{C} – constant fragment (of an antibody)
GP – glycoprotein
HDX-MS – hydrogen-deuterium exchange mass spectroscopy
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG - immunoglobulin G
IPTG - isopropyl β-D-1-thiogalactopyranoside

ITI – immune tolerance induction

LB – Luria-Bertani

MAD – multiple-wavelength anomalous dispersion

MES - 2-(N-morpholino)ethanesulfonic acid

MW – molecular weight

MWCO – molecular weight cut off

NTA – nitrilotriacetic acid

OD – optical density

PEG – polyethylene glycol

PDB – Protein Data Bank

PMSF - phenylmethanesulfonyl fluoride

pNPP – para-nitrophenylphosphate

PS - phosphatidylserine

RMSD – root mean square deviation

RPM – rotations per minute

SDS - sodium dodecyl sulfate

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC – size exclusion chromatography

SPR – surface plasmon resonance
TBS – Tris-buffered saline

TEV – tobacco etch virus

TIL – trypsin-inhibitor-like

Tris - 2-amino-2-hydroxymethyl-1,3-propanediol

Tris-HCL - 2-amino-2-hydroxymethyl-1,3-propanediol-hydrochloride

TRX – thioredoxin

v/v – volume/volume

vWD – von Willebrand’s disease

vWF – von Willebrand Factor
Background: Blood Coagulation and the Role of FVIII

Following vascular injury, excessive blood loss is prevented by the regulated reaction cascade of blood coagulation, or hemostasis. This highly conserved, multistep process acts rapidly at the site of tissue damage to halt potentially fatal bleeding. Hemostasis consists of three primary steps: vasoconstriction, platelet plug formation, and the formation of a fibrin clot. Vasoconstriction, or the narrowing of the blood vessels, is the first route to prevent blood loss, and is particularly key in slowing hemorrhage from arteries and large vessels. This is followed by the formation of a platelet plug, also known as a soft clot. Platelets are non-nucleate cells produced by megakaryocytes in the bone marrow that circulate in the bloodstream in their inactive form. Upon vascular damage, they undergo biochemical and morphological changes to become active, allowing them to adhere not only to each other, but also to exposed subendothelial collagen and subsequently form the initial clot. The final step in clot formation, the production of a fibrin mesh that supplements the aggregated platelets, is the result of a complex pathway known as the blood clotting cascade. In this process, a host of proteins known as clotting factors act in concert to ultimately produce insoluble fibrin from its soluble precursor fibrinogen, thus strengthening the clot and allowing normal wound healing processes to occur.
**Intrinsic vs. Extrinsic Pathway**

There are two pathways in coagulation that have different initial triggers but the same ultimate goal: the production of insoluble fibrin from its soluble precursor fibrinogen. Traditionally, the extrinsic pathway, or tissue factor pathway, is believed to be the most rapidly initiated pathway, responsible for an initial burst of thrombin activation, while the intrinsic pathway is necessary for a prolonged and sustained amplification of clotting activity. These two pathways converge on what is known as the final common pathway, in which thrombin is produced by activated factor X (fXa) and in turn cleaves fibrinogen into fibrin (Figure 1). Thus, it is only in the steps leading to the activation of fX to fXa that the pathways differ.

**Figure 1.** Blood coagulation cascade. The intrinsic pathway is shown in the red box, with the extrinsic pathway in the green. Both feed into the final common pathway and lead to the production of cross-linked fibrin mesh to supplement the platelet soft clot.
The extrinsic pathway begins with the exposure of an extravascular cell surface receptor called tissue factor to the bloodstream via vascular damage. This transmembrane protein serves as a cofactor to the serine protease fVII, which circulates constitutively in the bloodstream. Together, this complex activates the serine protease fX, either by a direct cleavage event, or by the intermediary path of activating fIX, which in turn can proteolytically activate fX. This leads to the common pathway processes of thrombin activation by fXa and subsequent formation of the insoluble fibrin mesh.

Table 1. Selected blood coagulation factors. Pathway, molecular weight, and function of plasma proteins are listed.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
<th>Pathway</th>
<th>MW (kDa)</th>
<th>Function</th>
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<tr>
<td>I</td>
<td>Fibrinogen</td>
<td>Both</td>
<td>340</td>
<td>Zymogen</td>
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<tr>
<td>II</td>
<td>Prothrombin</td>
<td>Both</td>
<td>72</td>
<td>Zymogen</td>
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<tr>
<td>III</td>
<td>Tissue Factor</td>
<td>Extrinsic</td>
<td>44</td>
<td>Cofactor</td>
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<tr>
<td>IV</td>
<td>Calcium Ions</td>
<td>Both</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>Proaccelerin; Labile Factor</td>
<td>Both</td>
<td>330</td>
<td>Cofactor</td>
</tr>
<tr>
<td>VII</td>
<td>Proconvertin; Stable Factor</td>
<td>Extrinsic</td>
<td>50</td>
<td>Cofactor</td>
</tr>
<tr>
<td>VIII</td>
<td>Antihemophilic Factor A</td>
<td>Intrinsic</td>
<td>285</td>
<td>Zymogen</td>
</tr>
<tr>
<td>IX</td>
<td>Christmas Factor; Antihemophilic Factor B</td>
<td>Intrinsic</td>
<td>57</td>
<td>Zymogen</td>
</tr>
<tr>
<td>X</td>
<td>Stuart-Prower Factor</td>
<td>Both</td>
<td>59</td>
<td>Zymogen</td>
</tr>
<tr>
<td>XI</td>
<td>Plasma Thromboplastin Antecedent (PTA)</td>
<td>Intrinsic</td>
<td>160</td>
<td>Zymogen</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman Factor</td>
<td>Intrinsic</td>
<td>76</td>
<td>Zymogen</td>
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<tr>
<td>XIII</td>
<td>Fibrin-stabilizing Factor</td>
<td>Both</td>
<td>320</td>
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By contrast, the intrinsic pathway, thus named because all of the components are already present internally in the bloodstream, begins with the activation of factor XII, which is initiated when this protein comes into contact with negatively charged surfaces.\(^6\) FXIIa is a serine protease that is responsible for the activation of FXI to FXIa, which in turn proteolytically activates FIX. Activated FIX forms a complex with factor VIIIa (FVIIIa), one of the non-protease members of the blood coagulation cascade, that binds activated platelet surfaces and serves as a cofactor to FXa in the proteolytic activation of FX (Figure 2). FXa forms a complex with its cofactor FVa, which has high sequence homology to FVIII and similarly serves to anchor the complex to the membrane.\(^7\) This complex is responsible for converting prothrombin into its active form, thrombin, which participates in a number of amplification feedback loops and ultimately cleaves soluble fibrinogen into insoluble fibrin. These fibrin monomers polymerize into the strong mesh that is responsible for supplementing the platelet plug to form a stable blood clot. The covalent cross-linking reaction results in the formation of an isopeptide bond between adjacent fibrin monomers and is catalyzed by active factor XIII (FXIIIa), which is also activated by the proteolytic action of thrombin.
Figure 2. Role of fVIII in blood coagulation. After dissociating from its circulatory partner, von Willebrand factor (vWF), fVIIIa binds to phosphotidylserine (PS)-expressing membranes and serves as a cofactor to serine protease fIXa in the proteolytic activation of fX.

**Factor VIII**

Coagulation fVIII is a 2,332-residue glycoprotein that is produced in multiple tissues in the body, including the liver, spleen, and kidneys. It is expressed with the domain arrangement of A1-A2-B-A3-C1-C2 prior to proteolytic processing and initially undergoes extensive glycosylation in the lumen of the endoplasmic reticulum and Golgi complex (Figure 3). The three A domains have been found to exhibit approximately 30%
sequence homology to each other as well as to the copper binding protein ceruloplasmin. The two C domains also have extensive homology to each other. In contrast, the B domain shows no known homology to other proteins. This domain undergoes proteolytic cleavage early in the fVIII life cycle at residues Arg1313 and Arg1648, thus forming a noncovalently associated heterodimer consisting of a heavy chain (A1-A2-B) and a light chain (A3-C1-C2) (Figure 4).

**Expression**

![Expression Diagram]

**Secretion**

![Secretion Diagram]

**Activation**

![Activation Diagram]

**Figure 3.** Domain arrangement of FVIII. While initially expressed as a single chain, following proteolytic processing of the B domain, FVIII is secreted as a heterodimer. The active form of the protein is a heterotrimer following cleavage by fXa or thrombin.

FVIII circulates in the bloodstream in this inactive form, noncovalently bound to the glycoprotein von Willebrand factor (vWF). This interaction prevents the breakdown of fVIII in circulation and is also thought to prevent premature binding of fVIII to fIXa and
activated platelet surfaces.\textsuperscript{15,16} Previous data suggests that two regions of the fVIII light chain are essential for vWF binding: the acidic region of the A3 domain corresponding to residues 1649-1689 and the carboxyl terminus of the C2 domain.\textsuperscript{17} Additionally, the fVIII light chain is also responsible for membrane binding, with the current working model indicating that solvent exposed, hydrophobic residues present on the beta hairpin loops of C2 embed within the membrane, while a ring of positively charged residues nearby interacts with the negatively charged phospholipid headgroup of phosphatidylserine (PS), thereby stabilizing the interaction.\textsuperscript{18}

\textbf{Figure 4.} X-ray crystal structure of B-domain deleted fVIII. Depicts the A1 (blue), A2 (cyan), A3 (orange), C1 (yellow), and C2 (red) domains.\textsuperscript{11}
Following vascular damage, fVIII undergoes proteolytic activation by thrombin or factor Xa to form a heterotrimer (A1/A2/A3-C1-C2).\textsuperscript{19,20} Thrombin cleaves fVIII at three sites, Arg1689 within the light chain and Arg372 and Arg740 in the heavy chain, while fXa cleaves at Arg336, Arg372, and Arg740.\textsuperscript{14} Activated fVIII (fVIIIa) dissociates from vWF and binds to activated platelet surfaces, where, in the presence of calcium ions, it serves as a cofactor for serine protease factor IXa. This complex, known as the intrinsic "tenase" complex, is responsible for converting fX to fXa at an increased rate of approximately 200,000-fold.\textsuperscript{19,21}
Part I Introduction: Hemophilia A and Inhibitory Antibodies

Hemophilia A

Hemophilia A is a blood clotting disorder caused by a lack of the blood coagulation protein factor VIII (fVIII). Congenital hemophilia A, which varies in severity depending on the amount of functional fVIII present, is an X-linked disorder affecting 1 in 5000 males worldwide.\textsuperscript{22,23} The disorder is characterized by frequent and prolonged bleeding episodes in which the blood fails to clot properly. Often, patients also suffer from excessive bruising and painful swelling in the joints due to accumulation of blood from internal bleeds. The severity of the disease is categorized according to the amount of functional fVIII present; mild hemophilia A is defined as 6-30% normal factor activity, moderate as 1-5%, and severe cases as less than 1% normal factor activity.\textsuperscript{24} The 186 kb gene encoding the fVIII protein, $F8$, is located on the long arm of the X-chromosome\textsuperscript{25} and is the site of over 2,500 unique mutations known to cause hemophilia A.\textsuperscript{26} Mutations leading to the disorder can occur throughout the gene and include intron inversions, insertions, deletions, nonsense and missense mutations.\textsuperscript{27, 28}
Hemophilia A Treatment

The primary treatment for the disease is infusions of functional fVIII.\textsuperscript{29,30} Previously, this fVIII was mainly human plasma-derived, but in recent years recombinant products have taken over as the principal source of fVIII for infusions. Patients with severe cases of hemophilia A often receive prophylactic infusions 2-3 times per week, as well as on-demand therapy for emergency situations. However, treatment is complicated by the development of neutralizing inhibitory antibodies directed against the infused fVIII. Approximately 30% of patients receiving replacement therapy develop inhibitory antibodies, an immune response leading to the clearance of fVIII from circulation and continued lack of clotting function, which persists long term.\textsuperscript{31-33} Although the immune response occurs in patients with mild to severe disease states, it has been noted that patients with more severe genetic lesions such as nonsense mutations, inversions, and large deletions that lead to the production of no endogenous fVIII often suffer from more severe immune response, possibly because the body does not recognize fVIII as self.\textsuperscript{34}

One treatment option used to overcome this inhibitory response is immune tolerance induction, or ITI. Although the exact protocols employed differ, the basis of ITI is in
giving patients very large doses of fVIII, sometimes accompanied by immunosuppressive drugs or other proteins, for prolonged periods of time to eventually abolish the immune response.\textsuperscript{35} As there is no one method of ITI that is effective for all patients, there are extensive differences in the dosing schedule and amount of fVIII needed to induce immune tolerance.\textsuperscript{36} Due to the high levels of fVIII often required, this treatment option is often cost prohibitive. Additionally, it is not successful in approximately 25\% of cases, leading to the ongoing need for additional treatment options for hemophilia A patients suffering extensive inhibitory response.\textsuperscript{37}

A number of strategies have been employed to produce less immunogenic fVIII that maintains a longer half-life in circulation. One such method is chemical modification in the form of PEGylation. The addition of polyethylene glycol polymers to therapeutic proteins has been shown to increase circulation time by “masking” the molecule from the immune system and reducing clearance by the kidneys.\textsuperscript{38} Fusion proteins represent another category of fVIII therapeutics currently in clinical trials. Two such strategies are the fusion of fVIII molecules to either the F\textsubscript{c} fragment of human immunoglobulin G (IgG) or to albumin, both of which increase half-life by binding to the neonatal F\textsubscript{c}-receptor, which prevents degradation and recycles the proteins back into circulation.\textsuperscript{39} Stabilization of fVIII via modification of the protein sequence itself is another tool for
increasing circulation half-life. Deletion of the B-domain coupled with covalent linkage of the heavy and light chains of the heterodimer via an engineered disulfide bond between the A2 and A3 domains was shown to increase stability by preventing the possibility of dissociation of the molecule at the A2 domain.\textsuperscript{40,41} Furthermore, it was demonstrated that mutations of certain residues at the interfaces of A2 with the A1 and A3 domains to decrease charge and increase hydrophobicity yielded FVIII molecules that were both more thermally stable and less prone to dissociation.\textsuperscript{42}

**FVIII and Inhibitory Antibodies**

In order to produce improved therapeutics, detailed knowledge of the structural interactions between FVIII and inhibitory antibodies is vital. First, a brief consideration of immunoglobulin G (IgG) structure is necessary. IgG molecules have a distinctive Y-shape that is composed of two heavy chains and two light chains, which are identical to each other respectively (Figure 5). There are two distinct types of fragments of the antibody, the $F_C$, or constant fragment, and the antigen binding fragment, $F_{AB}$. Each antibody contains two $F_{AB}$s, which are located at the tips of the molecule’s “arms” and are responsible for binding to the antibody’s target, or antigen. While the $F_C$ region of the antibody is conserved throughout its class, the $F_{AB}$ is highly variable, thus allowing it to bind to its target with exquisite specificity. Each $F_{AB}$ contains two variable domains, one
from the light chain and one from the heavy chain, each of which, in turn, contains three complementarity determining regions (CDRs), highly variable loops that recognize specific regions of the antigen called epitopes. The two F\textsubscript{AB}s can be separated from the F\textsubscript{C} domain through proteolytic cleavage by the enzyme papain, isolated from papaya, thus allowing for the study of antibody-antigen interactions using individual F\textsubscript{AB}s bound to their target proteins.

\textbf{Figure 5.} Schematic of antibody structure. The antibody consists of two heavy chains (dark blue) and two light chains (light blue), and is divided into an antigen binding fragment (F\textsubscript{AB}) and constant fragment (F\textsubscript{C}).
Characterization of the immune response to fVIII has revealed that the A2 and C2 domains are the sites of the majority of epitopes recognized by antibodies against fVIII. Antibodies against the C2 domain are broadly categorized into two classes based on their mechanism of inhibiting fVIII function. Classical antibodies are defined as those which block the binding of the C2 domain to vWF or phospholipid surfaces, which have been demonstrated to involve partially overlapping sites on C2. Conversely, non-classical antibodies prevent the activation of fVIII by thrombin or factor Xa and comprise the majority of fVIII C2 domain inhibitors.

Detailed structural studies of the C2 domain in complex with inhibitory antibodies have allowed the characterization of both classical and non-classical epitopes in recent years. A recent X-ray crystal structure of the C2 domain bound simultaneously to antigen binding fragments of both a classical and non-classical antibody demonstrated that these inhibitors bind to opposite faces of the protein (Figure 6).
Figure 6. X-ray crystal structure of the fVIII C2 domain ternary complex. The C2 domain is bound in complex with a classical antibody $F_{AB}$ (3E6) and non-classical $F_{AB}$ (G99), which bind on opposite faces of the C2 domain.\(^{49}\)

The classical antibody 3E6 was shown to sequester residues implicated in vWF binding, including Lys2183, Asp 2187, and Arg2215.\(^{52}\) The epitope of non-classical antibody G99, however, was demonstrated to consist partially of residues involved in binding to both factors IXa and Xa.\(^{52}\) While 3E6 was not shown to bind the solvent exposed hydrophobic loops implicated in phospholipid binding, the X-ray crystal structure of another classical antibody, BO2C11, in complex with C2 revealed that this inhibitor's variable region completely occludes the hydrophobic loops, preventing the key membrane binding function of C2.\(^{53}\) Thus, different antibodies falling under the same broad "classical" definition are seen to prevent C2 function in different ways. A 2014 study by Nguyen et al. used surface plasmon resonance (SPR) to map the epitopes of a panel of anti-C2 antibodies, building on previous work that defined subcategories of binding for classical
and non-classical inhibitors.\textsuperscript{54,47} Three distinct clusters of residues have been identified that correspond to five different antibody types, A, B, C, AB, and BC, which bind either exclusively to A, B, or C, or in an overlapping fashion to the AB or BC epitope regions.\textsuperscript{47}

In light of the C2-3E6-G99 ternary structure, some questions remain. Interestingly, as mentioned previously, the 3E6 epitope does not include the hydrophobic loops that are believed to embed in the phospholipid membrane upon fVIII binding. Thus, questions about how 3E6 exerts its neutralizing effects remain. One hypothesis presented by Walter et al. in the ternary structure paper is that long-range electrostatic changes occur upon antibody due to charge neutralization in the binding region.\textsuperscript{52} Additional structural data of the C2-E36 binding interactions could provide further insight into the mechanism of inhibition by this classical antibody. Aside from the interactions of 3E6 alone, unpublished data has suggested that the classical and non-classical antibodies bind cooperatively to their fVIII target. Subsequently, in hopes of elucidating some of these remaining structural questions, we aim to solve crystal structures of the binary complexes C2-G99 and C2-3E6 for comparisons with the C2-3E6-G99 ternary structure.
**X-ray Crystallography**

To study the structural characteristics of the fVIII protein complexes, X-ray crystallography was employed. X-ray crystallography is a structural technique that allows for the construction of an atomic-resolution model of macromolecular structure. The data for building these models is obtained from the diffraction of X-rays by many identical molecules in the ordered array of a crystal. These diffracted X-rays produce a pattern of spots, called reflections, on a detector. From this diffraction pattern, it is possible to determine the positions of the atoms that gave rise to the data. Specifically, because it is actually the electrons within the molecule that are scattering the X-rays, the diffraction pattern allows for the calculation of an electron density map into which can be modeled the amino acid residues from the known protein sequence.

The ability to produce an effective model using X-ray crystallography is contingent upon producing high quality protein crystals for diffraction. This process is often the limiting step of using X-ray crystallography to determine protein structure. In order to encourage proteins to crystallize, vapor diffusion is most commonly employed. In this technique, a drop of protein solution is mixed with a drop of buffer solution containing a precipitant and then allowed to equilibrate with a larger reservoir solution. Because the reservoir contains a comparatively higher concentration of the precipitant, water vapor...
slowly diffuses out of the protein droplet, thereby increasing the protein concentration in a gradual manner. Proteins, as with other molecules, can be encouraged to form crystals in supersaturated solution, which is facilitated by the vapor diffusion process.

A suitable crystal is then frozen in liquid nitrogen, placed into a monochromatic X-ray beam, and slowly rotated while being irradiated to collect a complete data set, which can then be used to generate an electron density map. The intensities of the waves scattered by the electrons in the crystal comprise the data that is measured. However, while the amplitude of these waves can be measured from the data, information regarding the phase of the waves is lost. This constitutes what is known in X-ray crystallography as "the phase problem." In order to solve the phase problem, three primary techniques are used. The first of these is multiple isomorphous replacement, in which heavy atoms derivatives of the protein are prepared and used to generate a diffraction pattern. Small perturbations in the heavy atom diffraction pattern versus that of the original crystal allow for the estimation of the phases. Another common phasing method is multiple-wavelength anomalous dispersion (MAD). This method also takes advantage of heavy atoms in the crystal, in this case by taking advantage of the heavy atoms' ability to absorb X-rays of certain wavelengths and then reemit the radiation with an altered phase, leading to an anomalous scattering signal. Finally,
there is molecular replacement, which has become the most common form of phasing. In this method, the crystallographer uses homologous structures to the protein under consideration to computationally determine the phases. Molecular replacement is the method employed in this study, as structures of the fVIII C2 domain and antibodies already exist. Following the determination of the phases, iterative cycles of model building and refinement can be used to construct a model that best fits the experimental data collected and elucidate structural questions.
Part 1: Research Aims

The goal of the presented research was to gain insights into the structural basis for interactions between the fVIII C2 domain and inhibitory antibodies. Specifically, we sought to elucidate the basis for antibody binding cooperativity that has been observed previously. To accomplish these goals, we first employed X-ray crystallography to determine the structure of the C2 domain bound in complex with classical inhibitory antibody 3E6.

Following determination of this binary complex structure, we compared this structure to a previously determined ternary structure of the C2 domain bound simultaneously to 3E6 and the non-classical inhibitory antibody G99. The objective of these structural comparisons was to ascertain if differences between the binary and ternary complexes could answer our questions about binding cooperativity and structural conservation among different complexes of C2 and inhibitory antibodies.
PART I: MATERIALS AND METHODS

C2-3E6 Complex Preparation

Purified 3E6 F_{AB} was incubated with a 1.5X molar excess of purified human C2 domain for 30 minutes at room temperature. Immediately after incubation, this sample was applied to HiLoad™ 16/60 Superdex 75 preparatory grade size exclusion chromatography column (120 mL column volume, GE Healthcare), which was attached to an AKTAprime Plus compact liquid chromatography system (GE Healthcare) and equilibrated in C2 low salt buffer (50 mM NaCl, 20 mM Tris-HCl (pH 8.0), 2.5% (v/v) glycerol). The eluent was collected and concentrated for SDS-PAGE analysis prior to X-ray crystallography trials.

Data Collection and Structure Determination

Hanging drop vapor diffusion was employed to produce crystals of adequate size for X-ray diffraction. Sparse matrix screens were optimized to encourage crystal growth. Crystals were grown in reservoir condition of 10mM MES, pH 6.5, and 20% (w/v) PEG 8000, and flash frozen using the same solution with the addition of 30% (v/v) DMSO as a cryoprotectant. X-ray diffraction data was collected at the Fred Hutchinson Cancer Research Center in Seattle, WA, on a Rigaku Micromax-007HF rotating anode with Confocal Varimax Optics Systems and an RAXIS-IV++ imaging plate detector. Diffraction
data was collected with CrystalClear (Rigaku), and indexing, scaling, and integration were accomplished with HKL2000. Initial phases were determined by molecular replacement with PHASER using the C2 domain (PBD: 1D7P), the variable region of the 3E6 F\textsubscript{AB} (PBD: 4KI5), and the constant region of the 3E6 F\textsubscript{AB} as search models. Model building and refinement were accomplished in iterative cycles with WinCoot and PHENIX, respectively.
Part I: Results

To further understand the complex interactions between the fVIII C2 domain and inhibitory antibodies, X-ray crystallography trials of the C2:3E6 binary complex were undertaken. Sparse matrix conditions were optimized to produce the final, successful result in 10 mM MES (pH 6.5) and 20% (w/v) PEG 8000. Crystals were allowed to grow by vapor diffusion over multiple months of incubation time. The successful data set, in which the crystal diffracted to 2.6 Å, was acquired following approximately one year of undisturbed incubation. Molecular replacement was used to solve the phase problem, taking advantage of previously solved structural information for both C2 and the 3E6 F\textsubscript{AB}. Following initial processing, it was determined that there were two copies of the complex (two C2 domains, each bound to a 3E6 F\textsubscript{AB}) present in the asymmetric unit. Thus, it was necessary to independently construct the two biological assemblies during the model building and refinement process.

Following the initial processing and phasing, model building and refinement were carried out in iterative cycles using the programs WinCoot and PHENIX, respectively.\textsuperscript{57,58} In this process, the model was examined residue by residue to identify areas of electron density that differed between the model and the experimental data. Adjustment of residue position or orientation was utilized to better fit the model to the data, as well as...
altering bond angles or individual rotamers (Figure 7). Each cycle of model building was then followed by one or more rounds of refinement using PHENIX. In later rounds of building, water molecules were modeled into areas of density present experimentally but not occupied by the model. Both experimental density and consideration of distances conducive to hydrogen bonding with nearby atoms were taken into account in the placement of 241 water molecules throughout the structure. Additionally, correction of Ramachandran outliers was undertaken as the model refinement continued. Following approximately 45 rounds of building and refinement, the model was completed and, at the time of writing, has been deposited into the Protein Data Bank (PDB) pending publication, with the PDB ID 4XZU.

**Figure 7.** Density Map. Example of an electron density map and corresponding residues from WinCoot during the model building process.
Refinement statistics of R-work and R-free were monitored throughout the refinement process. R-work, also known as R-factor, is a measure of the quality of the model. During the refinement process, the atomic model is used to calculate an electron density map, which is then compared to the experimentally determined electron density. The R-work describes how closely these two are in agreement. However, this process introduces an element of bias in that the atomic model and electron density are used to calculate the new electron density. The circularity of this process can lead to over-fitting of the model. To counter this bias and assess the validity of the model, another statistic, known as R-free, is calculated. R-free is calculated in the same manner as R-factor but uses a small subset of residues that are set aside and not used in refinement. R-free is then a measure of how well the current model predicts the experimental data from this portion of the data set that is unbiased. While the R-free is often significantly higher than the R-work early in the model building process, they should become more similar as refinement is accomplished.
Table 2. Crystallographic data and refinement statistics from C2:3E6 structure.

<table>
<thead>
<tr>
<th>Data Collection Statistics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>1.54</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>39.77 - 2.609 (2.702 - 2.609)</td>
</tr>
<tr>
<td>Space group</td>
<td>P 2 2_1 2_1</td>
</tr>
<tr>
<td>Unit cell (Å, °)</td>
<td>a = 43.23, b = 148.49, c = 188.42, α = β = γ = 90</td>
</tr>
<tr>
<td>Total reflections</td>
<td>211,017</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>36,886 (3,537)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.7 (5.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.86 (93.47)</td>
</tr>
<tr>
<td>Mean I/sigma(I)</td>
<td>15.20 (6.08)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>48.03</td>
</tr>
<tr>
<td>R-merge</td>
<td>0.067 (0.292)</td>
</tr>
</tbody>
</table>

| Refinement Statistics               |       |
| Resolution (Å)                      | 40 – 2.61 (2.68 – 2.61) |
| R-work                              | 0.1988 (0.2500) |
| R-free                              | 0.2673 (0.3491) |
| Number of non-hydrogen atoms        | 9,034 |
| macromolecules                      | 8,790 |
| ligands                             | 3 |
| water                               | 241 |
| Protein residues                    | 1,137 |
| RMS bonds (Å)                       | 0.011 |
| RMS angles (°)                      | 1.36 |
| Ramachandran                        |       |
| favored (%)                         | 91 |
| allowed (%)                         | 8.2 |
| outliers (%)                        | 0.8 |
| MolProbity Clashescore              | 12.62 |
| Average B-factor (Å²)               | 49.4 |
| macromolecules                      | 49.6 |
| ligands                             | 70.1 |
| solvent                             | 41.1 |
| PDB code                            | 4XZU |
The final values of R-work and R-free for the C2:3E6 structure were lowered to 0.1988 and 0.2673, respectively, from initial values of 0.2500 and 0.3491. The following additional model validation parameters were also verified: bond root mean squared deviation (RMSD), angle RMSD, clashscore, and average B-factors. Bond RMSD describes the root mean squared deviation of the bond lengths from accepted values. Similarly, angle root mean squared deviation is a measure of how angle distances differ from standard values. The clashscore describes the overlap of nearby atoms’ electron density, with greater than 0.4 Å overlap calculated as a clash by PHENIX. The “average B” parameter represents an average of the B factors within the model to allow for a more globalized comparison. B-factors, also called temperature factors, describe the scattering of electrons by each atom due to thermal motion. In general terms, it is used as a description for the dynamic mobility of each atom in the structure. An unusually high B-factor can also be an indicator of an error in model building, which is the reasoning behind an examination of B-factors in model validation. The average B for the C2 3E6 structure was 49.4 Å² overall; individual B-factor values will be discussed further below.

Following the completion of the model, detailed analysis of the structure was undertaken (Figure 8). The C2 domains from each of the two complexes in the
asymmetric unit were well-resolved and consist of fVIII residues Cys2174-Glu2327 with no omissions within these residues. The C2:3E6 binding interfaces for each of the two complexes were similarly well-resolved and able to be modeled unambiguously into the electron density. Of the two 3E6 F\textsubscript{Abs}, that from one of the complexes (designated Complex 1 and consisting heavy chain A and light chain B in the model) was well defined except for heavy chains residues 132-133, which were omitted from the final model. The F\textsubscript{Ab} from the second complex (Complex 2, consisting of heavy chain E and light chain F in the model) was less well defined and contained a number of areas of poor electron density, specifically the absence of residues 130-135 and 177 from the heavy chain and 106, 149-154, 187-191, and 198-203 from the light chain. Importantly, as noted above, none of these residues were near the binding interface, which was the focus of most structural comparisons.
Figure 8. X-ray crystal structure of the C2-3E6 binary complex A) Both copies of the complex in the asymmetric unit, including their orientation during the modeling process. The C2 domains of each biological assembly are shown in red and blue, while their corresponding F\textsubscript{AB}s are shown in magenta and cyan, respectively B) 90° rotation from the view in (A), showing the orientation of each assembly with respect to the other.

Interestingly, a comparison of the two copies of the complex within the asymmetric unit reveals that while considerable overlap exists between the structures, the F\textsubscript{AB} elbow angle, which separates the constant and variable portions of the F\textsubscript{AB}, is significantly different between Complex 1 and Complex 2 (Figure 9).
Figure 9. Overlay figure of the two copies of C2:3E6 in the asymmetric unit (blue and red, respectively) with the C2:3E6 portion of the ternary structure (green). The dramatic difference in elbow angle between the two copies of the binary complex is illustrated in (A), while (B) emphasizes the overall structural conservation between the binary and ternary complex models.

This variation, while not uncommon for $F_{\text{AB}}$ structures, is a fairly dramatic example of the dynamic flexibility of antibody structure that allows them to bind targets with such exquisite precision. Aside from this angle variation, overlay of the structures reveals that the variable regions of Complex 1 and Complex 2 are highly conserved, particularly in the C2:3E6 interface. Furthermore, very little difference is noted between the binding interface of each binary complex and that of the previously determined C2:3E6:G99 ternary complex. Following superposition of the binary and ternary complexes, the
RMSD of the Cα atoms between C2:3E6:G99 and binary complexes 1 and 2 were calculated to be 0.328 and 0.383 Å, respectively.

A closer examination of the C2:3E6 interface reveals that the antibody epitope is discontinuous, with 3E6 sequestering two loops of the C2 domain, largely consisting of residues Glu2181-Ala2188 and Thr2202-Arg2215. Of these two loops, seven residues seem poised to make direct protein-antibody contacts based on distances. These residues are Lys2183, Asp2187, Arg2209, His2211, Gln2213, Gly2214, and Arg2215 (Figure 10). Of these residues Lys2183, which is within hydrogen bonding distance of Trp90, Tyr93, and Ser91 of the FAB, and Gln2213, which is similarly near to FAB residues Ser33, Thr30, and Thr53, are particularly occluded by antibody binding. The 3E6 FABs from the binary complexes bury a similar amount of surface area to that of the C2:3E6:G99 ternary structure, which was calculated to be 690 Å² by Walter et al.\textsuperscript{52}
As noted above, comparison of the C2:3E6 binary structure with the C2:3E6:G99 binary structure is surprising not for any structural changes but for the lack thereof: the two structures show remarkable similarity when overlaid. Interactions detailed above for the C2:3E6 binary structure are similarly demonstrated in the ternary complex. As in the ternary structure, residues Lys2183 and Asp2187, each of which is believed to be important for vWF binding, are occluded by the binding of 3E6, which is in keeping with the definition of 3E6 as a classical antibody, as these inhibitors interfere with C2 binding of vWF or phospholipid surfaces. One interesting difference between key interface residues, however, is the positioning of Arg2215. As seen in Figure 11, in the ternary

Figure 10. Overlay figure of C2:3E6 binary and C2:3E6:G99 ternary complexes showing key residues at the C2-3E6 interface. Colors: Green, ternary complex; blue/cyan, binary complex 1; and red/magenta, binary complex 2. Right: interactions between Asp2187 and Arg2209 with 3E6 residues are conserved in both binary and ternary complexes. Left: His2211 through Arg2215 are part of a C2 loop that is strongly occluded by 3E6 binding.
structure this residue makes two key salt bridges with Asp100 of 3E6. However, in the
two copies of the binary structure, we see that in Complex 1 (shown in blue), only one
of these salt bridges is maintained due to differences in Arg2215 position, while in the
other binary structure, Complex 2 (shown in magenta), neither of these interactions is
maintained.

Figure 11. Overlay figure of C2:3E6 binary and C2:3E6:G99 ternary complexes showing
variation in the position of Arg2215. In the ternary complex (green), Arg2215 makes two
salt bridges with Asp100 of the 3E6 FAB. In complex 1 of the binary complexes (blue),
only one of the salt bridges is possible due to the altered position of the residue, while
in complex 2 (magenta), neither interaction is possible.

Upon determination of the ternary structure, Walter et al. noted that, surprisingly, 3E6
does not occlude the hydrophobic loops containing Met2199-Phe2200 and Leu2251-
Leu52, which are believed to be key for anchoring C2 into the phospholipid
membrane.⁵² This is seen as well in the binary structure, which recognizes a
discontinuous epitope that does not include these residues. Walter et al. proposed that, rather than direct interaction with these loops, long-range electrostatic changes upon antibody binding inhibit proper association of C2 with the membrane.

The considerable similarities between the C2:3E6 binary structure and the C2:3E6:G99 ternary structure suggest that the binding cooperativity noted previously between the G99 and 3E6 antibodies (P. Lollar and S.L. Meeks, unpublished results) is not the result of drastic conformational changes in the binding interface. This leads to the consideration of other possible contributors to this cooperative behavior, specifically electrostatic or dynamic changes. To understand electrostatic contributions, pKa calculations for the C2 domain alone versus in complex with 3E6 were accomplished with PROPKA.\textsuperscript{60} This revealed that most of the significant perturbations (>0.25 pH units) occurred in residues composing the 3E6 epitope, which is unsurprising given the interactions these residues make with the antibody. Only two residues, His2269 (-0.4 pH units) and His2315 (-0.6 pH units), near the G99 epitope exhibited significant pKa perturbations. This indicates a weaker case for pKa changes as the basis of cooperativity than dynamic changes (see below), although it is not ruled out entirely.
Table 3. PKa changes upon binding. Examples of pKa changes of ionizable groups in C2 alone versus in the C2:3E6 binary complex predicted using PROPKA.60

<table>
<thead>
<tr>
<th>Residue</th>
<th>Location</th>
<th>pKa Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu2182</td>
<td>3E6 Epitope</td>
<td>2.6</td>
</tr>
<tr>
<td>Glu2322</td>
<td>3E6 Epitope</td>
<td>1.3</td>
</tr>
<tr>
<td>Lys2183</td>
<td>3E6 Epitope</td>
<td>-2.4</td>
</tr>
<tr>
<td>His2211</td>
<td>3E6 Epitope</td>
<td>-1.2</td>
</tr>
<tr>
<td>Arg2215</td>
<td>3E6 Epitope</td>
<td>1.8</td>
</tr>
<tr>
<td>His2269</td>
<td>G99 Epitope</td>
<td>-0.4</td>
</tr>
<tr>
<td>His2315</td>
<td>G99 Epitope</td>
<td>-0.6</td>
</tr>
</tbody>
</table>

To explore the possible contributions of dynamics to the cooperativity question, an examination of B-factors was undertaken. As mentioned previously, B-factors can be used to describe the dynamic mobility of atoms in a structure. In order to compare B-factors between multiple structures of the fVIII C2 domain, however, normalization to the overall B-factors for each structure was necessary, as B-factors are crystal-dependent. To this end, averages were determined for the localized B-factors of each loop and then divided by the previously described “average B” value for each structure. This process was undertaken for C2 domain loops from C2:3E6 binary complexes 1 and 2, the C2:3E6:G99 ternary complex, the C2:BO2C11 complex, and isolated the C2 domain.
As expected, B factors for the loops composing the C2:3E6 interface were lower than the corresponding values for C2 alone (Figure 12). For the Gln2213-Ser2216 loop, an average of the B factor ratios from the binary and ternary complexes was 0.91 versus 1.50 for isolated C2 domain (Table 2). This loop also partially composes the epitope of classical antibody BO2C11 and has a likewise reduced B factor ratio value from that structure of 0.88. Similarly, the second major loop of the 3E6 epitope, Glu2181-Gln2189, upon antibody binding has a B factor ratio of 0.81 (averaged from the binary, ternary, and BO2C11 complexes) versus 1.07 for the C2 domain alone. Thus, we see that the B factors of loops involved in antibody binding are generally lower than those of the C2
alone, which agrees with the loss of dynamic motion following binding. Interestingly, three loops that partially compose the G99 epitope, Ser2265-Trp2271, Phe2275-Lys2279, and Val2223-Glu2228, also display lowered B factor ratios in the C2:3E6 binary complex structure. As revealed by the C2:3E6:G99 ternary structure, one residue in particular, Lys2227, is key to the binding of G99 to C2. The loop that contains this residue, Val2223-Glu2228, has calculated B factor ratios of 0.83, 0.99, and 1.08, respectively, for the ternary complex, binary complex 1, and binary complex 2, while the value calculated for C2 alone was 1.23.

Table 4. B-factor ratios for comparison of B-factors at different epitopes amongst multiple C2:antibody protein complexes.

<table>
<thead>
<tr>
<th>C2 epitope</th>
<th>C2 ternary</th>
<th>C2 binary#1</th>
<th>C2 binary#2</th>
<th>C2/BO2C11</th>
<th>C2 alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>2181-2189</td>
<td>0.82</td>
<td>0.80</td>
<td>0.73</td>
<td>0.88</td>
<td>1.07</td>
</tr>
<tr>
<td>2197-2201</td>
<td>1.27</td>
<td>1.32</td>
<td>0.96</td>
<td>0.80</td>
<td>1.56</td>
</tr>
<tr>
<td>2213-2216</td>
<td>1.00</td>
<td>0.85</td>
<td>0.87</td>
<td>0.89</td>
<td>1.52</td>
</tr>
<tr>
<td>2223-2228</td>
<td>0.84</td>
<td>0.99</td>
<td>1.08</td>
<td>1.33</td>
<td>1.23</td>
</tr>
<tr>
<td>2265-2271</td>
<td>1.07</td>
<td>1.49</td>
<td>1.26</td>
<td>1.05</td>
<td>1.63</td>
</tr>
<tr>
<td>2275-2279</td>
<td>1.24</td>
<td>1.11</td>
<td>1.41</td>
<td>1.56</td>
<td>1.39</td>
</tr>
</tbody>
</table>

One other loop of note that had lowered B factor ratios was Thr2197-Ala2201, which, while not part of either the 3E6 or G99 epitope, is both a key component of the BO2C11 epitope and is suggested as important for membrane binding. Furthermore, a previous
study found, via H-D exchange data, that this loop has increased protection factors upon 3E6 binding.\(^6\) Subsequently, it seems reasonable to propose that 3E6 binding decreases dynamic mobility of not only loops comprising the 3E6 epitope, but also loops present as other regions of the C2 domain surface that are involved in non-classical antibody interactions and membrane binding. The decrease in dynamic motion of these loops upon 3E6 binding may serve to lower the entropic cost of binding a second antibody, thus providing a basis for the cooperative binding noted between 3E6 and G99. Ultimately, while questions still exist with regards to this cooperative nature, the elucidation of structures such as C2:3E6 will hopefully provide a greater wealth of knowledge for understanding the complex fVIII-antibody interactions and lead to the development of improved therapeutics for patients suffering from hemophilia A.
Part II Introduction: FVIII and vWF

As noted in the first part of this document, recent X-ray crystal structures have helped elucidate the complex interactions between fVIII and inhibitory antibodies, while also allowing for greater refinement of the proposed mechanism of the fVIII C2 domain's membrane binding role. However, one piece of the fVIII puzzle that still remains poorly understood is detailed information about the interactions of fVIII with its circulatory partner, von Willebrand factor (vWF). Interestingly, it has been noted in some studies that plasma-derived fVIII concentrates containing vWF resulted in lower levels of inhibitory antibody response in patients receiving these infusions.\textsuperscript{62-64} This indicates a protective effect on fVIII by vWF, possibly stemming from the masking of epitopes by the presence of vWF.\textsuperscript{65,66} While there is still intense debate over the merits of plasma-derived versus recombinant fVIII with regards to immune tolerance,\textsuperscript{34} intriguing questions remain over the role of vWF and its interactions with fVIII. Detailed structural information about the binding of vWF to fVIII would be a helpful tool in elucidating the interactions between these two proteins.

In addition, the fVIII-vWF pairing is at the heart of another bleeding disorder that, while not as prominent as hemophilia A, is the most common inherited bleeding disorder in the world: von Willebrand's disease (vWD).\textsuperscript{67,68} In this disorder, which often manifests
with significantly more mild symptoms than hemophilia, the blood fails to clot properly
due to a lack of vWF in the body.\textsuperscript{69-71} In severe cases, vWD can be mistaken for
hemophilia A, as the lack of vWF can lead to dangerously low plasma levels of fVIII, since
this protein is rapidly degraded by proteases when not bound to vWF in circulation.\textsuperscript{72,73}

von Willebrand's disease was first described in the 1920s by Finnish physician Erik von
Willebrand, for whom it was later named. von Willebrand characterized the disease
after studying the case of a young girl from Finland's Aland islands whose family had an
extensive history of hemorrhagic issues.\textsuperscript{74} The isolated nature of the island population
meant that there was a high incidence of this inherited disease, which is autosomal
recessive, unlike hemophilia A, and thus equally affects men and women. Today, it is
believed that vWD affects anywhere from 1 in 100 to 1 in 1000 individuals, though many
will never know they have the disease because the symptoms are so mild.\textsuperscript{67} For most
cases, the primary treatment is the on-demand administration of desmopressin, which
stimulates the release of vWF.\textsuperscript{75} In the event of severe bleeds, however, IV infusions of
vWF are often necessary.

There are three different types of vWF, depending on whether the disorder is
quantitative or qualitative in nature. Type 1 vWD is a quantitative disorder in which the
body produces abnormally low levels of vWF. It is the most common form of the disease, accounting for approximately 75% of cases and is generally mild. The third form, Type 3 vWD is also a quantitative disorder, in which the individual is homozygous for the defective gene, and the body fails to produce any vWF, subsequently leading to extremely low levels of fVIII. It is the rarest form of the disease but also the most severe. The second form, Type 2 vWD, is a qualitative disorder that has 4 subcategories: 2A, 2B, 2M, and 2N. Each of these subcategories is caused by a different gene mutation that affects vWF's ability to associate with its various binding partners. Type 2N, or the Normandy type, is caused by the inability of vWF to bind to fVIII, thus leading to hemophilia A-type levels of fVIII in circulation.

**von Willebrand Factor**

von Willebrand factor (vWF) is a 2,050 residue glycoprotein that is produced in endothelial cells and megakaryocytes. It is initially synthesized as a 2,813 residue product that contains a 22 amino acid signal peptide and 741 residue propeptide that is eventually removed by proteolytic cleavage in the Golgi apparatus. Characterization of the domains from early cDNA sequence work gives the arrangement of D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-C1-C2-CTCK (Figure 13). However, more recent examination of sequence and structure relationships indicates that the protein is highly mosaic with
complex assemblies of modules lacking hydrophobic cores but connected through extensive disulfide bonding.\textsuperscript{88} The three A domains, which are the only domains without an abundance of cysteines, have been thoroughly characterized and their structures determined, revealing them to be large and globular. The D domains are now understood to consist of smaller modules, denoted as vWD domains, cysteine-8 (C8) domains, trypsin-inhibitor-like (TIL) domains, and E domains, based on sequence and structural homology studies.\textsuperscript{88} The B and C domains have been re-categorized as 6 tandem von Willebrand C (VWC) and VWC-like domains, and are followed by the c-terminal cysteine knot (CTCK) domain, which is homologous to the cysteine knot superfamily of proteins.\textsuperscript{88}

![Figure 13. Domain arrangement of vWF.](image)

Upon entry into the endoplasmic reticulum early in their life cycle, provWF monomers form "tail-to-tail" dimers through disulfide linkages between their CTCK domains.\textsuperscript{89,90}
Then, following extensive glycosylation that is proposed to occur prior to the dimerization step, the provWF dimers are then transported to the Golgi apparatus where they undergo sulfation of oligosaccharides at Asn384 and Asn468, additional glycosylation, and proteolytic cleavage of the propeptide (domains D1 and D2).\textsuperscript{81,91,92} They then undergo extensive multimerization through "head-to-head" disulfide linkages involving their D3 domains, and can form large multimers that are greater than 20,000 kDa in size.\textsuperscript{81} These large multimers are necessary for proper vWF function. While some vWF is secreted constitutively, the rest is stored in granules in endothelial cells called Weibel-Palade bodies until release upon vascular injury.\textsuperscript{93}

\textbf{Figure 14.} Depiction of vWF’s role in blood clotting. Large vWF multimers help recruit platelets to the site of vascular injury.
vWF is not an enzyme but instead performs a number of functions through binding to other proteins. In the event of injury, vWF binds to subendothelial collagen through its A3 (and possibly A1) domain and once immobilized then binds inactive platelets under conditions of high fluid shear stress (Figure 14).\textsuperscript{94-97} It binds these platelets through interaction of the A1 domain with the platelet glycoprotein (GP) Ib receptor complex and thus recruits them to the site of damage.\textsuperscript{98} Additionally, vWF can also bind to activated platelets through the integrin $\alpha_{\text{IIb}}$$\beta_3$ binding to its C1 domain.\textsuperscript{99}

Aside from platelet adhesion at the site of vascular injury, vWF also plays another key role in the blood coagulation process by serving as the circulatory partner of fVIII and preventing its breakdown in circulation. It is estimated that 95% of fVIII in circulation is in complex with vWF, which protects it from proteolytic degradation and is also thought to prevent premature association with fIXa and activated platelet surfaces.\textsuperscript{100,101} The importance of this interaction is highlighted by the severity of Type 3 and Type 2N vWD, in which the body produces normal fVIII levels but often maintains fVIII concentrations in the blood at near hemophilia A levels due to the inability of fVIII to bind vWF.

The primary binding fVIII binding site on vWF was located to the N-terminal 272 residues by Foster et al. in 1987 and was further confirmed to be harbored in the D'D3 domains
in following studies.\textsuperscript{15,17} Recent evidence suggests that the D' domain, which is itself composed of two regions, the trypsin-inhibitor-like, or TIL', and E' domains, is primarily responsible for binding to fVIII.\textsuperscript{102} Epitope mapping studies using anti-vWF monoclonal antibodies, as well as the fact that 72\% of missense mutations leading to Type 2N vWF are located in the TIL' domain, make a strong case for this as the primary binding site.\textsuperscript{103,104} Furthermore, a recent solution structure of the TIL'E' domains by Shiltagh et al. provides additional evidence, with the authors proposing a binding model in which positive charge in the TIL' region, supported by an inflexible scaffold of the E' region and rigid TIL' \( \beta \)-sheets, forms electrostatic attractions with the acidic region of the fVIII A3 domain that has been demonstrated to be critical for vWF binding (Figure 15).\textsuperscript{102} This acidic region, along with the C2 domain, have been shown to comprise the primary vWF binding site on fVIII.\textsuperscript{17,105,106}
Figure 15. Solution structure of the TIL'E' domains, which are suggested to harbor the primary fVIII binding site on vWF.\textsuperscript{102} These domains are also known collectively as D'.

Despite this evidence, however, no structures exist depicting fVIII bound to vWF. The complex arrangement and high cysteine content of vWF has stymied acquisition of detailed structural information for all but the A domains of the protein. In this study, we attempt co-crystallization of fVIII in complex with the D'D3 domains and with the smaller binding fragment D', which will be hereafter referred to by the new naming convention of TIL'E' when referred to in isolation (not part of the D'D3 fragment).
Part II: Research Aims

The goals of this research were threefold. First, we aimed to express and purify the vWF TIL’E’ (D’) domain for use in structural studies with fVIII and domain mutants thereof. Following purification, we proposed to form complexes between TIL’E’ and fVIII, between TIL’E’ and the fVIII C2 domain, and between TIL’E’ and an 18-residue oligopeptide corresponding to a region of the fVIII a3 domain thought to be essential for fVIII:vWF binding. Furthermore, we aimed to form these complexes with a second, larger vWF domain fragment, D’D3, in place of the TIL’E’ to further elucidate fVIII:vWF binding. To confirm formation of these complexes, affinity pull-down assays were devised, supplemented by enzyme-linked immunosorbent assays (ELISA) to further assess binding.

The third aim of this research was to employ X-ray crystallography to study binding interactions between fVIII and the vFW domain fragments. To this end, X-ray crystal trials were initiated through the application of hanging-drop vapor diffusion.
PART II: Materials and Methods

Buffers

TIL'E’ Wash Buffer: 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 40 mM imidazole

TIL'E’ Elution Buffer: 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 200 mM imidazole

TIL'E’ Storage Buffer: 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5% glycerol

FVIII Storage Buffer: 20 mM HEPES (pH 7.5), 5 mM CaCl₂, 350 mM NaCl

D'D3 Storage Buffer: 25 mM Tris-HCl (pH 8.0), 100 mM NaCl
Expression of TIL'E

A pET32b+ vector encoding the TIL'E' domain of vWF was acquired from the Hanson lab of University College, London. The plasmid also encoded an N-terminal thioredoxin (TRX) tag and N-terminal internal (His)\textsubscript{6}-tag, separated from the coding region by a 31-residue linker and tobacco etch virus (TEV) protease cleavage site. The vector was transformed into New England Biosciences SHuffle\textsuperscript{®} cells, and colonies were selected from ampicillin selection plates for large-scale expression. Ten mL Luria Bertani (LB) broth (10\% (w/v) BactoTryptone, 5\% (w/v) yeast extract, 10\% (w/v) NaCl) cultures containing 50 mg/mL ampicillin (AMP) were inoculated from selected colonies and allowed to grow overnight at 37°C. These starter cultures were then used to inoculate 1 L LB/AMP cultures, which were grown at 37°C until an OD\textsubscript{600} reading of 0.6 was reached and then induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The temperature was lowered to 15°C and cultures were allowed to grow overnight with shaking. The following day, cells were pelleted by centrifugation at 6,300 x g for 10 minutes in a Sorvall GS-3 rotor (Du Pont Instruments) and stored at -20°C until purification.
Affinity Chromatography Purification of TIL'E

Cell pellets were resuspended in 30-mL TIL'E' wash buffer. Lysozyme and phenylmethanesulfonyl fluoride (PMSF) were added to final concentrations of 1 mg/mL and 1 mM, respectively, and the solution was incubated with gentle agitation for 30 minutes at 4°C. Cells were then lysed via sonication in two 45-second intervals (Branson sonifer, 50% duty cycle). The lysate was centrifuged at 36,000 x g for 40 minutes in a Sorvall SS-34 rotor (Du Pont Instruments) to pellet insoluble cell debris, and the resulting supernatant was filtered prior to chromatography through successive 5 µm and 0.45 µm filters. HisPur™ Ni-NTA affinity resin (Thermo Scientific) was pre-equilibrated in TIL'E' wash buffer prior to incubation with the filtered lysate for one hour with stirring at 4°C. The solution was then applied to a gravity flow column, and the resin washed with 10 column volumes (cv) of TIL'E' wash buffer. (His)_6-tagged TIL'E' was eluted using with TIL'E' elution buffer, with successive 2-mL fractions monitored for presence of protein using Bradford reagent. Fractions were then pooled and dialyzed into TIL'E' storage buffer overnight at 4°C using 12-14 molecular weight cut off (MWCO) dialysis tubing (Spectrum Laboratories, Inc.).
**TEV Cleavage**

Tobacco etch virus (TEV) protease was used to cleave the TRX-(His)$_6$-tag-linker fragment from the TIL'E' domain prior to further studies. A ratio of 1 OD$_{280}$ unit TEV to 5 OD$_{280}$ units TIL'E' was utilized for the cleavage reactions. Samples containing (His)$_6$-tagged TEV and TIL'E' were incubated at room temperature for three hours prior to overnight incubation at 4°C. The following day, samples were applied to a gravity flow column containing Ni-NTA affinity resin pre-equilibrated in TIL'E' wash buffer and incubated for 30 minutes to encourage binding of (His)$_6$-tagged TEV and cleavage fragments to the metal ions. The initial flow through and 5-mL wash with TIL'E' wash buffer were collected prior to washing the column with TIL'E' elution buffer. A fraction of this eluent was collected for SDS-PAGE analysis to determine of cleavage efficiency. The initial fractions collected were buffer-exchanged into TIL'E' storage buffer and concentrated using an Amicon Ultra-15 Centrifugal filters (Ultracell 10K membrane, Millipore) prior to SDS-PAGE analysis.

**SEC of TIL'E'**

As a final purification step, cleaved TIL'E' was applied to a HiLoadTM 16/60 Superdex 75 preparatory grade size exclusion chromatography column (120 mL column volume, GE Healthcare). The column was pre-equilibrated in TIL'E' storage buffer and attached to an
ÄKTApriime Plus compact liquid chromatography system (GE Healthcare). TIL'E' eluent and impurity peaks were collected for SDS-PAGE analysis.

**Binding Assay**

To assess the binding of the vWF TIL'E' and D'D3 domains to fVIII, an affinity resin pull-down assay was employed. A 100 µL sample containing 50 µg of (His)_6-tagged TIL'E' or D'D3 and an equimolar ratio of fVIII ET3 in TIL'E' or D'D3 storage buffer was incubated at room temperature for 15 minutes. This sample was then incubated for 10 minutes at room temperature with 100 µL of 50% HisPur™Ni/NTA resin (Thermo Scientific) slurry equilibrated in TIL'E' or D'D3 storage buffer. Following incubation, the sample was transferred to a Costar Spin-X® centrifuge tube filter (Corning Incorporated) and centrifuged at 500 x g for 1 minute. The initial flow through was collected prior to the resin being washed 2X for 1 minute intervals at 500 x g with 250 µL of storage buffer. Protein was then eluted in an additional centrifugation step with 100 µL TIL'E' elution buffer. Flow through, wash, and elution samples were then analyzed via SDS-PAGE.

**Formation of vWF:fVIII Complexes**

Purified TIL'E' or D'D3 was mixed with fVIII ET3 in a 1.5:1 molar excess of the vWF domain to fVIII. Samples were incubated at room temperature for 15 minutes to
encourage complex formation. Complexes were then concentrated to >1 mg/mL using Pierce 3K MWCO concentrators (Thermo Scientific) for X-ray crystallography trials.

**X-ray Crystal Trials**

Hanging drop vapor diffusion was selected to crystallize the fVIII:D’D3 or fVIII:TIL'E' complexes. Initial screening for suitable crystallization conditions was achieved with sparse matrix screens from Hampton Research and Rigaku Reagents. In this method, 400 µL of each condition was pipetted into the reservoirs of 24-well plates. One µL of well solution was placed on a glass cover slide and mixed with 1 µL of protein complex. The cover slide was then immediately placed over the well, which was pre-greased to provide a strong seal upon placing of the slide. Trays were left undisturbed for 24-hours to allow vapor diffusion to take place prior to checking for the presence of crystal formation. Initial hits from sparse matrix screens were optimized by varying pH, salt, precipitant, and protein: mother liquor ratios in order to grow larger and more well-defined crystals for diffraction.

**Enzyme-linked Immunosorbant Assays (ELISA)**

Purified fVIII, fVIII:D’D3, or fVIII:TIL'E' was incubated in rows A-C and E-G of 96-well plates for 45 minutes at 37°C. Plates were then rinsed 3X with 1X Tris-buffered saline
(TBS) solution. Rinsed plates were blocked with 3X bovine serine albumin (BSA) for 1 hour at 37°C and again rinsed 3X in TBS. From starting samples of 1200 nM, twelve part serial dilutions of 3E6 and G99 IgG were made in 1% (w/v) BSA/TBS and plated in rows A-D and E-H, respectively, in the blocked and rinsed plates. Plates were then incubated at 37°C for 90 minutes, prior to rinsing 3X in TBS. Goat anti-mouse 2° antibody conjugated to alkaline phosphatase (AP) (Kirkegaard and Perry Laboratories, Inc.) was diluted 1:2000 in 1X TBS and was then incubated in all wells for 30 minutes at 37°C before a final rinsing step (3X in 1X TBS). AP substrate p-nitrophenyl phosphate (pNPP) was then added to all wells and incubated for approximately 5 minutes at 37°C prior to reading absorbance at 405 nm on a BioTek Epoch plate reader.

**FVIII Acidic Region Peptide Binding Assays**

To assess binding to the vWF domain fragments and act as a crystallization aid, an 18-residue peptide of the sequence MKKEDFDIYDEDENQPRS, corresponding to residues 1691-1709 of fVIII was constructed. This peptide was produced by solid phase peptide synthesis in the Antos Lab of Western Washington University, and contained an N-terminal biotin tag for experimental purposes. To test binding of this construct to vWF domain fragments TIL’E’ and D’D3, a simple resin binding assay similar to that reported above was utilized. Following a 15 minute, room temperature incubation of a 1:1 ratio
of peptide and either D’D3 or TIL’E’, 100 µL of streptavidin-agarose resin (50% slurry, Thermo Scientific) was added to the sample and incubated with gentle agitation for 10 minutes at room temperature to encourage binding. These samples were then added to spin filters and centrifuged as described previously, with initial flow through, two wash, and elution fractions collected for SDS-PAGE analysis.
Part II: Results and Discussion

Preparation of Pure TIL'E' Domain

For X-ray crystallography studies, a relatively large amount of purified protein (10-100 mg) is required. To this end, expression and purification of the vWF TIL'E' domain was initiated. A bacterial expression system was utilized, with transformation of the TIL'E' plasmid into the New England Biosciences SHuffle E. coli line. This cell line is engineered to aid in the proper folding of disulfide-bonded proteins in the cytoplasm of the cell, containing a mutation that allows for the constitutive production of disulfide bond isomerase DsbC. Additionally, to encourage the proper folding of disulfide bonds within the TIL'E' domain, the TIL'E' plasmid contains an N-terminal thioredoxin (TRX) protein tag (Figure 16).

<table>
<thead>
<tr>
<th>TRX</th>
<th>His₆-tag</th>
<th>31-residue linker</th>
<th>TEV</th>
<th>TIL'E'</th>
</tr>
</thead>
</table>

Figure 16. Schematic depicting arrangement of the TIL'E' (D')-TRX fusion protein encoded by the pET32b(+) vector. TRX, thioredoxin tag; His₆-tag, hexahistidine tag; TEV, tobacco etch cleavage virus protease cleavage site.

Following protein expression, three column chromatography steps were utilized to ensure sufficient purity for crystal trials. The initial Ni/NTA affinity chromatography step takes advantage of the internal (His)₆-tag encoded by the pET15b(+) vector to separate
the majority of impurities from the TIL'E' domain (Figure 17). This was followed by
removal of TRX and the (His)_6-tag by tobacco etch virus (TEV) protease cleavage.
Another Ni/NTA chromatography step was used to remove the (His)_6-tagged TEV, TRX-
(His)_6-tag-linker fragment, and any uncleaved TIL'E' from the purified fraction (Figure
18). Finally, to remove trace impurities, a final size-exclusion chromatography step was
employed (Figure 19).

**Figure 17.** SDS-PAGE analysis of TIL'E' (D') protein purity following initial affinity metal
chromatography purification step. Lanes: 1. Molecular weight marker; 2. Filtered lysate;

Following each column chromatography step, the purity of the protein was assessed by
SDS-PAGE. Prior to cleavage of the tags, the TIL'E' fusion protein migrates near 27 kD.
After the cleavage step, the final mass of the TIL'E' domain alone is 11 kD. While
contaminating bands are seen on all of the gels following initial purification steps, after
the final SEC step, a single band at the expected molecular weight for TIL'E' is present.
**Figure 18.** SDS-PAGE analysis following TEV cleavage of TIL'E’ (D’). Lanes: 1. Molecular weight marker; 2. Uncleaved TIL'E’; 3. TEV + TIL'E'; 4. Ni/NTA wash fraction; 5. Ni/NTA elution fraction.

**Figure 19.** Analysis of TIL'E’ (D’) purity following size-exclusion chromatography. Left, elution profile of TIL'E’. Right, SDS-PAGE after chromatography step. Lanes: 1. Molecular weight marker; 2; Pre-SEC TIL'E’ sample; 3. Pooled impurity peaks fraction; 4. TIL'E’ peak.
Formation of fVIII:TIL'E' and fVIII:D'D3 Complexes

The fVIII:TIL'E' and fVIII:D'D3 complexes were formed by incubation of a 1.25:1 ratio of vWF domain: fVIII for 15 minutes at room temperature. Due to the large size of the complex, it was not an ideal candidate for in-house size exclusion chromatography, as it would elute in the void volume of the HiLoad™ 16/60 Superdex 75 preparatory grade size exclusion chromatography column. Therefore, we devised a simple assay to confirm binding of the vWF domains to fVIII. Taking advantage of the (His)_6-tag present on both TIL'E' and D'D3 prior to cleavage, a Ni/NTA affinity pull-down protocol was developed, in which a sample containing fVIII complexed with tagged-vWF domain was incubated with Ni/NTA resin and then centrifuged. Initial flow through, wash, and elution fractions were checked for the presence of proteins by SDS-PAGE. The fVIII and vWF domains were present in the elution fraction, indicating that the fVIII bound to the tagged D'D3 or TIL'E' and was not simply washed away in the initial flow through or subsequent washing steps (Figure 20).

Attempt to Form HC2:TIL’E’ and HC2:D’D3 Complexes

Previous studies have indicated that while the primary vWF binding site on fVIII consists of both the acidic region of the fVIII light chain and the C2 domain, the C2 domain alone can bind to full-length vWF with a 564 nM affinity.\textsuperscript{17} As the primary fVIII binding site on vWF has been located to the N-terminal D’D3 region, we wanted to assess the binding of the human fVIII C2 domain to the D’D3 fragment and to TIL’E’. The same Ni/NTA affinity pull-down assay was utilized as described above, but with (His)\textsubscript{6}-tagged C2 and untagged D’D3 or TIL’E’ (Figure 21). The results of this assay were somewhat inconclusive. In only one of the five trials with C2:TIL’E’ was TIL’E’ detected in the elution profile; in all
remaining trials it was seen instead in the initial flow through step. The C2:D’D3 complex had similar results, in which only one of the trials indicated that binding had occurred. This indicates that the binding interaction between C2 and TIL'E' or D'D3 was often not sufficiently strong to survive the centrifugation step, as weak binding was seen only infrequently in the repetitions of the assay.

![Figure 21. SDS-PAGE analysis of C2 binding to D’D3. Lanes: 1. Molecular weight marker; 2. C2; 3. D’D3; 4. Initial flow through; 5. Wash #1; 6. Wash #2; 7. Elution.](image)

Following this initial result, size exclusion chromatography was utilized to assess binding. Following a 30 minute incubation at room temperature to encourage complex formation, C2:TIL'E' (not shown) or C2:D'D3 was applied to a size exclusion chromatography column but repeatedly failed to co-elute (Figure 22). We were not able
to form stable C2:TIL’E’ or C2:D’D3 complexes for crystallographic studies based on the methods of detection we had at our disposal.


**FVIII Acidic Region Peptide**

As noted above, previous studies have identified the fVIII acidic region as partially comprising the vWF binding site. Particularly, residues 1669-1689 of the a3 region have been shown to be a strong determinant for binding. To investigate this interaction, a biotinylated 18-residue peptide was synthesized, consisting of residues 1671-1689. As peptides have been shown to be aids for crystallization of proteins resistant to
formation of crystals, it was hoped that formation of a complex between the peptide and the D’D3 or TIL’E’ domains would encourage these proteins to crystallize.

To test binding of the peptide to the vWF domains, a simple resin binding assay was utilized. This assay took advantage of the N-terminal biotin tag present on the peptide, as biotin forms a very strong interaction with streptavidin. Using streptavidin-agarose resin in place of Ni/NTA, a binding assay similar to those described previously was employed for probing peptide:D’D3 or peptide:TIL’E’ binding (Figure 23). Surprisingly, the vWF domains were seen in the initial flow through and wash fractions, and failed to be detected in the sample containing the streptavidin-agarose beads, even though association of the peptide with streptavidin using biolayer interferometry (BLI) measurements indicated that the biotin tag was binding as expected. This result indicates that the acidic region peptide is not sufficient for binding to these isolated vWF fragments strongly enough to be detected by an affinity pull-down assay.
Figure 23. SDS-PAGE analysis of lack of binding of fVIII acidic region peptide to D'D3. Lanes: 1. Molecular weight marker; 2. D'D3; 3. Initial flow through; 4. Wash #1; 5. Wash #2; 6. Elution.

**ELISA**

To probe the interaction between fVIII and the vWF domain fragments, enzyme-linked immunosorbent assays (ELISAs) were undertaken. Studies focused on immune tolerance induction (ITI) as a therapy for hemophilia A have suggested that vWF plays a protective role in abrogating the immune response of inhibitory antibodies generated against fVIII. As discussed in the introduction, two inhibitory antibodies against fVIII, classical antibody 3E6 and non-classical antibody G99, have been shown to target the C2 domain. As the C2 domain is implicated as a part of the vWF binding site on fVIII, we were interested in what effect the presence of D'D3 or TIL'E' would have on the binding of either 3E6 or G99 to fVIII. In the ELISA assays, the fVIII:TIL'E' or fVIII:D'D3 complex was adhered to the 96-well plate, followed by incubation with 3E6 or G99 IgG. A secondary
antibody, conjugated to alkaline phosphatase, targeting the Fc region of the IgGs was then used for detection of IgG binding.

Both G99 and 3E6 IgGs were able to bind fVIII even in the presence of D’D3 or TIL’E’ (Figure 24). In the presence of TIL’E’, the binding dissociation constant, or \( K_d \), was 2.3 nM for the 3E6 IgG and 1.4 nM for the G99 IgG. These values are comparable to those of the IgGs binding to FVIII alone, which were 2.3 nM and 2.8 nM, respectively, for 3E6 and G99. Similar numbers were obtained for the presence of D’D3. For the 3E6 IgG, a \( K_d \) value of 3.8 nM was found, whereas the G99 IgG had a \( K_d \) of 0.66 nM. Thus, while small variations in the \( K_d \) values were noted, no significant reduction in IgG binding was seen in the presence of the vWF domains.
Figure 24. ELISA data of IgG binding to fVIII in the presence of TIL’E’ (D’) or D’D3. Top, no vWF domains. Middle, IgG binding to fVIII:D’D3 complex. Bottom, IgG binding to fVIII:TIL’E’ complex. Open circles: fVIII + 3E6, solid squares: fVIII + G99. Each figure is the result of two repeated experiments completed in triplicate.
Binding of F\textsubscript{AB}s to fVIII in Presence of vWF Domains

Following these ELISA results comparing the F\textsubscript{AB} binding to fVIII in the presence of vWF domains, we were interested in confirming this outcome using another method. We employed resin binding assays as described previously but with one additional step: following the incubation of fVIII with either TIL’E’ or D’D3, G99 or 3E6 F\textsubscript{AB} was added to the sample in a 1:1 molar ratio with fVIII. The sample was then incubated at room temperature for 15 minutes before addition of the Ni/NTA resin and subsequent centrifugation steps. The presence of SDS-PAGE gel bands corresponding to the F\textsubscript{AB}s in the elution step indicates that, regardless of the presence of TIL’E’ or D’D3, the antibodies are still able to bind fVIII (Figures 25 and 26). This corroborates the findings from the ELISA assays.

**Figure 25.** SDS-PAGE analysis of binding of F\textsubscript{AB}s to fVIII in the presence of TIL’E’ (D’). Left, binding of 3E6 F\textsubscript{AB} to fVIII. Lanes: 1. Molecular weight marker; 2. fVIII; 3. TIL’E’; 4. 3E6; 5. Initial flow through; 6. Wash #1; 7. Wash #2; 8. Elution. Right, binding of G99 F\textsubscript{AB} to fVIII. Lanes: 1. Molecular weight marker; 2. fVIII; 3. TIL’E’; 4. G99; 5. Initial flow through; 6. Wash #1; 7. Wash #2; 8. Elution.

X-ray Crystal Trials

X-ray crystallography provides a powerful tool for elucidating the structural interactions in protein complexes. To examine the interactions in fVIII:vWF binding more thoroughly, X-ray crystal trials involving the fVIII:D’D3 complex and fVIII:TIL’E’ complex were initiated. Over 2,000 sparse matrix conditions were screened for each complex. Table 3 identifies the screens used to identify initial crystal leads, purchased from Hampton Research and Rigaku Reagents.
Table 5. List and brief descriptions of sparse matrix screens utilized for crystallization attempts of fVIII complexes.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Manufacturer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal Screen</td>
<td>Hampton Research</td>
<td>Primary, highly diverse sparse matrix screen, utilizing a wide variety of salts and precipitants over a wide pH range.</td>
</tr>
<tr>
<td>Index</td>
<td>Hampton Research</td>
<td>Primary, highly diverse sparse matrix screen, utilizing a wide variety of salts and precipitants over a wide pH range.</td>
</tr>
<tr>
<td>PEG/Ion</td>
<td>Hampton Research</td>
<td>Sparse matrix screen evaluating range of salt conditions with monodisperse polyethylene glycol precipitants.</td>
</tr>
<tr>
<td>PEG Rx</td>
<td>Hampton Research</td>
<td>Sparse matrix screen evaluating different polymer precipitants over a wide pH range.</td>
</tr>
<tr>
<td>Crystal Screen Cryo</td>
<td>Hampton Research</td>
<td>Sparse matrix screen evaluating range of buffers and salts against cryoprotectants, including glycerol, PEG 400, and others.</td>
</tr>
<tr>
<td>Wizard Classic</td>
<td>Rigaku Reagents</td>
<td>Primary, highly diverse sparse matrix screen, utilizing a wide variety of salts and precipitants over a wide pH range.</td>
</tr>
<tr>
<td>Precipitant Synergy</td>
<td>Rigaku Reagents</td>
<td>Sparse matrix screen utilizing multiple precipitants simultaneously in a single condition to encourage crystallization in difficult protein targets.</td>
</tr>
<tr>
<td>PEGs Suite</td>
<td>Quiagen</td>
<td>Sparse matrix screen employing a wide pH range with low salt concentrations against a variety of polyethylene glycol polymers as precipitants.</td>
</tr>
</tbody>
</table>
FVIII: TIL'E' Complex

The FVIII: TIL'E' complex yielded multiple leads from sparse matrix conditions. In each of these cases, the crystals were generally flat and diamond-shaped in appearance, lacking the 3-dimensionality needed for successful diffraction (Figure 27). Additionally, the more 3D crystals were composed of thin, overlapping sheets, as opposed to a single, thick layer. Four of these conditions were selected for optimization to encourage growth of larger crystals with better morphology for diffraction (Table 4). The optimization process generally consists of varying two components of the crystal conditions, one in the 24-well plate’s X-direction and one in the Y-direction, while keeping the others constant. Frequently, these changes involve altering the concentration or identity of a salt, precipitant, or buffer component. For example, a common strategy is the varying of the polyethylene glycol (PEG) concentration in the X-direction, while varying the solution pH in the Y-direction.

Table 6. Sparse matrix conditions yielding leads for fVIII:TIL’E’ (fVIII:D’) complex.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Manufacturer</th>
<th>Initial Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG/Ion 2 #35</td>
<td>Hampton Research</td>
<td>2% v/v Tacsimate, 0.1 M Tris (pH 8.5), 16% w/v PEG 3350</td>
</tr>
<tr>
<td>PEG/Ion 2 #45</td>
<td>Hampton Research</td>
<td>0.1 M CsCl, 15% w/v PEG 3350</td>
</tr>
<tr>
<td>Crystal Screen 2 #30</td>
<td>Hampton Research</td>
<td>0.1 M HEPES (pH 7.5), 10% w/v PEG 6000, 5% v/v MPD</td>
</tr>
<tr>
<td>Index #61</td>
<td>Hampton Research</td>
<td>0.2 M proline, 0.1 M HEPES (pH 7.5), 10% w/v PEG 3350</td>
</tr>
</tbody>
</table>
Following optimization, three crystals, listed in Table 5, were selected for screening at the Fred Hutchinson Cancer Research Center in Seattle to ascertain if they diffracted. These crystals were cryoprotected by the addition of 30% DMSO and immediately flash frozen. Of the three crystals, one diffracted very weakly. While not strong enough diffraction for the collection of a data set, this result identified the crystals as protein rather than salt, and indicated that the fVIII:TIL’E’ complex is able to be crystallized, which is a considerable hurdle in the X-ray crystallography process. At the time of this
writing, optimizations of the fVIII:TIL’E’ crystallization conditions are ongoing in the hopes of producing crystals with stronger diffraction.

Table 7. List of fVIII:TIL’E’ (fVIII:D’) crystals selected for screening at the Fred Hutchinson Cancer Research Center in Seattle, WA.

<table>
<thead>
<tr>
<th>Initial Screen</th>
<th>Optimization #</th>
<th>Buffer</th>
<th>Salt(s)/Additives</th>
<th>Precipitant</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG/Ion 2 #35</td>
<td>1</td>
<td>0.1 M Tris-HCl (pH 6.9)</td>
<td>2% Tacsimate (pH 8.0)</td>
<td>16% PEG 3350</td>
</tr>
<tr>
<td>PEG/Ion 2 #35</td>
<td>1</td>
<td>0.1 M Tris-HCl (pH 7.25)</td>
<td>2% Tacsimate (pH 8.0)</td>
<td>18% PEG 3350</td>
</tr>
<tr>
<td>PEG/Ion 2 #45</td>
<td>1</td>
<td>None</td>
<td>0.05 M CsCl</td>
<td>17% PEG 3350</td>
</tr>
</tbody>
</table>

FVIII: D’D3 Complex

Initial sparse matrix conditions with the fVIII:D’D3 complex yielded less leads than the fVIII:TIL’E’ complex. None of the seven conditions with initial hits resulted in the formation of relatively large, distinct crystals as in the fVIII:TIL’E’ trays (Figure 28). However, two conditions, PEG R_x 2 #27 and #43, yielded showers of crystalline precipitate that were selected for optimization, while 3 conditions, Wizard Screen #3, #5 and #74, resulted in the formation of quasi-crystals, which were circular in shape with no clearly defined edges. One condition, PEG/Ion 2 #47, produced a single, defined crystal, but this was not able to be reproduced in following trials. Table 6 lists the initial leads selected for optimization. While crystallization in all conditions except for PEG/Ion
2 #47 was able to be recapitulated, none of the conditions yielded significant improvement in size or morphology of the initial leads. Ongoing optimization is needed to improve upon initial fVIII:D’D3 complex leads.

Table 8. Sparse matrix conditions yielding hits for the fVIII:D’D3 complex.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Manufacturer</th>
<th>Buffer</th>
<th>Salt(s)/Additives</th>
<th>Precipitant(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG/Ion 2</td>
<td>Hampton Research</td>
<td>0.05 M HEPES (pH 7.0)</td>
<td>1% Tryptone</td>
<td>12% (w/v) PEG 3350</td>
</tr>
<tr>
<td>#47</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PEG Rx 2</td>
<td>Hampton Research</td>
<td>0.1 M Tris-HCl (pH 8.0)</td>
<td>2% 1,4-Dioxane</td>
<td>15% (w/v) PEG 3350</td>
</tr>
<tr>
<td>#27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG Rx 2</td>
<td>Hampton Research</td>
<td>0.1 M HEPES (pH 7.5)</td>
<td>N/A</td>
<td>10% (w/v) PEG 10,000 5% MPD</td>
</tr>
<tr>
<td>#43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wizard #3</td>
<td>Rigaku Reagents</td>
<td>0.1 M CHES (pH 9.5)</td>
<td>N/A</td>
<td>15% (v/v) Reagent Alcohol</td>
</tr>
<tr>
<td>Wizard #5</td>
<td>Rigaku Reagents</td>
<td>0.1 M CAPS (pH 10.5)</td>
<td>N/A</td>
<td>30% (v/v) PEG 400</td>
</tr>
<tr>
<td>Wizard #74</td>
<td>Rigaku Reagents</td>
<td>0.1 M CHES (pH 9.5)</td>
<td>N/A</td>
<td>30% (v/v) PEG 400</td>
</tr>
<tr>
<td>PEGs #77</td>
<td>Quiagen</td>
<td>N/A</td>
<td>0.2 M Potassium Acetate</td>
<td>20% (w/v) PEG 3350</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>
Figure 28. Images of crystals obtained in sparse matrix screens of the fVIII:D’D3 complex
Conclusions

The importance of fVIII and its interactions with other proteins is exemplified by the bleeding disorders engendered by its absence. Both hemophilia A and type 2N von Willebrand's disease present a lifetime's worth of complications for patients, with costly and inconvenient treatments an unfortunate necessity. Subsequently, better understanding of the structural basis of fVIII's interactions with both inhibitors and its circulatory binding partner, von Willebrand factor, is needed in order to move forward with the development of superior treatments and patient care.

As noted above, recent years have seen an increase in the structural understandings of fVIII's interactions with inhibitory antibodies. The 2.6 Å resolution structure of the fVIII C2 domain in complex with classical inhibitory antibody 3E6 further supports the findings from the recent C2-3E6-G99 ternary structure. Comparisons between these two structures reveal considerable overlap between residues at the C2-3E6 interface, indicating that the 3E6 epitope on C2 is well-conserved both in the absence and presence of non-classical inhibitory antibodies. As noted previously for the ternary structure, the 3E6 F\textsubscript{AB} appears to occlude residues on the C2 domain thought to be important for von Willebrand factor binding.
Interestingly, however, ELISA and resin binding studies indicate that 3E6 is still able to bind to fVIII in the presence of vWF domain fragments thought to contain the primary fVIII binding site. Furthermore, while earlier studies have demonstrated that the C2 domain alone is able to weakly bind full-length vWF, the formation of stable complexes between either D'D3 and C2 or TIL'E' and C2 for X-ray crystallography was not achieved. Thus, we hypothesized that the role of C2 in the vWF binding site on fVIII was not the major determinant of stable binding.

The publication of two recent studies involving the binding of vWF to fVIII sheds light on these findings and indicates an exciting new direction in the field of structural interactions between fVIII and its binding partner. The first of these studies, by Yee et al., presents low-resolution cryo-electron microscopy data of the vWF D'D3 domains binding to fVIII. A structure based on 3D reconstruction of these data presents a novel binding model, in which the D' module of vWF interacts with fVIII via the C1 domain primarily, as opposed to a connection through the C2 domain (Figure 29). The authors emphasize the dynamic nature of this interaction, in part due to the inability to fit the fVIII C domains into the data envelope as readily as the more static A domains. This may indicate significant conformational changes in the C domains upon binding to vWF. The
second recent publication involving fVIII-vWF interactions further emphasizes the role of the fVIII C1 domain in binding. Chiu et al. performed negative-stain electron microscopy (EM) and hydrogen-deuterium mass spectroscopy (HDX-MS) studies with D’D3 in an attempt to elucidate the primary vWF binding site on fVIII. Their findings corroborate those of Yee et al. in that the fVIII C1 domain is established as the major vWF binding site. However, while Yee et al. were unable to definitively establish a role for the acidic region of the fVIII A3 domain in vWF binding—which had been previously indicated as critical for fVIII-vWF association—Chiu et al. determined by HDX-MS that structural perturbations in the a3 region indicate the involvement of these residues in binding.

Figure 29. Model of vWF D’D3 domain binding to fVIII proposed by Yee et al. following collection of low resolution electron microscopy data.

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These recent studies support our findings that neither the C2 domain alone nor the acidic region peptide were sufficient to establish stable complexes with the vWF D'D3 domains. Furthermore, this launches an exciting new direction in the structural studies of fVIII and vWF, as our focus shifts from the C2 domain alone to the C1 domain and C1-C2 multi-domain region. Production of the C1 domain in isolation in bacterial expression systems has, to our knowledge, thus far not been achieved. However, the expression system of the pET32b(+) vector in the NEB SHuffle cell line has shown great promise in production of the previously intractable vWF TIL'E' domain. Therefore, attempts are underway to utilize this system for the production of isolated C1 and C1-C2 domain constructs, with the ultimate goal of using X-ray crystallography to elucidate the detailed structural mechanism by which the FVIII C1 domain binds to vWF and thereby add to the body of fVIII structural knowledge. While the intricacies of fVIII's interactions with other proteins in circulation have often eluded researchers, recent structural findings will hopefully help pave the way for greater understanding of these interactions and, subsequently, improved therapeutic strategies for patients suffering from fVIII-related diseases.
References


